FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)
209861US0PCT	09/868338

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP99/07079	16 December 1999	18 December 1998

TITLE OF INVENTION
ABC TRANSPORTER AND GENE CODING FOR THE SAME

APPLICANT(S) FOR DO/EO/US
Sohei KANNO, et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☒ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Request for Consideration of Documents Cited in International Search Report
Notice of Priority
PCT/IB/304, PCT/IB/308
Sequence Listing (10 Pages)

JC03 Rec'd PCT/PTO 18 JUN 2001

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/868338	PCT/JP99/07079	209861US0PCT

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1000.00	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$860.00	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$710.00	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$690.00	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =		$860.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	14 - 20 =	0	x $18.00	$0.00	
Independent claims	7 - 3 =	4	x $80.00	$320.00	
Multiple Dependent Claims **(check if applicable)**.			☐	$0.00	
TOTAL OF ABOVE CALCULATIONS =				$1,180.00	
☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				$0.00	
SUBTOTAL =				$1,180.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				$0.00	
TOTAL NATIONAL FEE =				$1,180.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				$0.00	
TOTAL FEES ENCLOSED =				$1,180.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$1,180.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **15-0030** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Surinder Sachar
Registration No. 34,423

Surinder Sachar
SIGNATURE

Norman F. Oblon
NAME

24,618
REGISTRATION NUMBER

6-18-01
DATE

DOCKET NO.: 209861US-0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :

Sohei KANNNO et al : ATTN: BOX SEQUENCE

SERIAL NO: 09/868,338 :

FILED: June 18, 2001

FOR: ABC TRANSPORTER AND GENE CODING FOR THE SAME

PRELIMINARY AMENDMENT AND STATEMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Responsive to the Notice to Comply dated July 23, 2001, Applicants submit herewith amendments to the specification, a substitute Sequence Listing, and a corresponding Computer-Readable Sequence Listing.

IN THE SPECIFICATION

Please replace the paragraph beginning on page 4, line 25, through page 5, line 11, with the following paragraph:

--PCR (polymerase chain reaction) is performed by using chromosomal DNA of *Brevibacterium lactofermentum* such as *Brevibacterium lactofermentum* ATCC13869 as a template and primers having nucleotide sequences of regions in the *g1tBD* genes of *Escherichia Coli* K-12 (*Gene, vol. 60*, pp. 1-11 (1987) and yeast (*Saccharomyces cerevisiae*, GenBank Accession No. X89221) exhibiting high homology, for example, those having nucleotide sequences of SEQ ID NOS: 1 and 2 shown in Sequence Listing, to obtain a DNA

fragment of about 1.4 kb. *Brevibacterium lactofermentum* ATCC13869 can be obtained from ATCC (the American Type Culture Collection: 10801 University Boulevard, Manassas, VA 20110-2209, United States of America).--

Please replace the paragraph on page 9, line 24, through page 10, line 26, with the following paragraph:

--A DNA coding for substantially the same protein as a constituent of ABC transporter can be obtained by expressing DNA having such a mutation as described above in an appropriate cell, and examining characteristics of an expressed product. A DNA coding for substantially the same protein as a constituent of ABC transporter can also be obtained by isolating a DNA hybridizable with a nucleotide sequence coding for each constituent or a probe prepared from such a nucleotide sequence, for example, the nucleotide sequence of nucleotide numbers 1117 to 1725 in SEQ ID NO: 7 or a probe prepared from this nucleotide sequence, for ATPase under a stringent condition, and coding for a protein having the characteristics of the constituent from a DNA coding for each protein having mutation or from a cell harboring it. The "stringent condition" referred to herein is a condition under which a so-called specific hybrid is formed, but a non-specific hybrid is not formed. It is difficult to clearly define this condition by using numerical values. However, for example, the stringent condition includes a condition under which two of DNAs having high homology, for example, two of DNAs having homology of not less than 60% are hybridized with each other, but two of DNAs having homology lower than the above level are not hybridized with each other. Alternatively, the stringent condition is exemplified by a hybridization condition represented by salt concentrations of 1 x SSC, 0.1% SDS, preferably

0.1 x SSC, 0.1% SDS, at 60°C, which is an ordinary condition of washing in Southern hybridization.--

Page 26 (Abstract), after the last line, beginning on the next page, please replace page 27 to page 36 of the original Sequence Listing with the substitute Sequence Listing attached herewith.

IN THE CLAIMS

Please amend the claims as follows:

--4. (Amended) The DNA according to Claim 3, wherein the stringent condition is a condition in which hybridization is performed at 60°C and a salt concentration corresponding to 1 x SSC and 0.1% SDS.

8. (Amended) The DNA according to Claim 7, wherein the stringent condition is a condition in which hybridization is performed at 60°C and a salt concentration corresponding to 1 x SSC and 0.1% SDS.

12. (Amended) The DNA according to Claim 11, wherein the stringent condition is a condition in which hybridization is performed at 60°C and at a salt concentration corresponding to 1 x SSC and 0.1% SDS.

REMARKS

Claims 1-14 are active in the present application. Claims 4, 8 and 12 have been amended. Support for the amendment is found, for example, on page 10, lines 12-26. The specification has been amended to correct typographical or clerical errors.

Applicants have now submitted a substitute Sequence Listing and a corresponding Computer-Readable Sequence Listing. Contents of the paper copy of the substitute Sequence Listing and the Computer-Readable Sequence Listing are identical. Support for all the sequences listed in the substitute Sequence Listing can be found in the present application. No new matter is introduced by the submission of the substitute Sequence Listing and the Computer-Readable Sequence Listing.

Applicants submit that this application is now in condition for examination on the merits. Early notice to this effect is earnestly solicited.

Respectfully submitted,

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MARKED-UP COPY OF
PRELIMINARY AMENDMENT AND STATEMENT

IN THE SPECIFICATION

Please replace the paragraph beginning on page 4, line 25, through page 5, line 11, with the following paragraph:

--PCR (polymerase chain reaction) is performed by using chromosomal DNA of *Brevibacterium lactofermentum* such as *Brevibacterium lactofermentum* ATCC13869 as a template and primers having nucleotide sequences of regions in the *g1tBD* genes of *Escherichia Coli* K-12 (*Gene, vol. 60*, pp. 1-11 (1987) and yeast (*Saccharomyces cerevisiae*, GenBank Accession No. X89221) exhibiting high homology, for example, those having nucleotide sequences of SEQ ID NOS: 1 and 2 shown in Sequence Listing, to obtain a DNA fragment of about 1.4 kb. *Brevibacterium lactofermentum* ATCC13869 can be obtained from ATCC (the American Type Culture Collection: [12301 Parklawn Drive, Rockville, Maryland, 20852] 10801 University Boulevard, Manassas, VA 20110-2209, United States of America).--

Please replace the paragraph on page 9, line 24, through page 10, line 26, with the following paragraph:

--A DNA coding for substantially the same protein as a constituent of ABC transporter can be obtained by expressing DNA having such a mutation as described above in an appropriate cell, and examining characteristics of an expressed product. A DNA coding for substantially the same protein as a constituent of ABC transporter can also be obtained by

isolating a DNA hybridizable with a nucleotide sequence coding for each constituent or a probe prepared from such a nucleotide sequence, for example, the nucleotide sequence of nucleotide numbers 1117 to 1725 in SEQ ID NO: 7 or a probe prepared from this nucleotide sequence, for ATPase under a stringent condition, and coding for a protein having the characteristics of the constituent from a DNA coding for each protein having mutation or from a cell harboring it. The "stringent condition" referred to herein is a condition under which a so-called specific hybrid is formed, but a non-specific hybrid is not formed. It is difficult to clearly define this condition by using numerical values. However, for example, the stringent condition includes a condition under which two of DNAs having high homology, for example, two of DNAs having homology of not less than [40%] 60% are hybridized with each other, but two of DNAs having homology lower than the above level are not hybridized with each other. Alternatively, the stringent condition is exemplified by a hybridization condition represented by salt concentrations of 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C, which is an ordinary condition of washing in Southern hybridization.--

IN THE CLAIMS

--4. (Amended) The DNA according to Claim 3, wherein the stringent condition is a condition in which [washing] hybridization is performed at 60°C and a salt concentration corresponding to 1 x SSC and 0.1% SDS.

8. (Amended) The DNA according to Claim 7, wherein the stringent condition is a condition in which [washing] hybridization is performed at 60°C and a salt concentration corresponding to 1 x SSC and 0.1% SDS.

12. (Amended) The DNA according to Claim 11, wherein the stringent condition is a condition in which [washing] hybridization is performed at 60°C and at a salt concentration corresponding to 1 x SSC and 0.1% SDS.--

SPECIFICATION

ABC TRANSPORTER AND GENE CODING FOR THE SAME

Technical Field

The present invention relates to a novel ABC transporter and a gene coding for a protein that is a constituent of the ABC transporter. The gene can be utilized for breeding of a microorganism showing modified transport of amino acids across a cell membrane and so forth.

Background Art

There are several mechanisms are known for transport of substances such as an amino acids or ions through cell membranes. As one of such mechanisms, the ATP-binding cassette superfamily (ABC transporters) is known (C.F. Higgins, *Ann. Rev. Cell Biol.*, *8*, 67 (1992)).

The ATP-binding cassettes constitute a group of proteins having an ATP-binding domain including a transmembrane domain. Their physiological function is primarily uptake of substances into a cell, but the ATP-binding cassette is considered to also participate in excretion of substances to some extent. In bacteria, they usually contain, as constituents, membrane proteins

(membrane components), proteins that are present inside the membrane and have the ATPase activity, and binding proteins that are present outside the membrane and bound to substances. The membrane proteins and proteins having the ATPase activity form a polymer complex. It is said that the substance excretion system lacks a binding protein bound to a substance to be transported (Reizer, J. *et al.*, *Prot. Sci. 1*, 1326 (1992)).

Since the ABC transporters or constituents thereof are involved in transport of substances, it is considered that characteristics of a cell concerning substance transport can be modified by modifying expression of genes coding for them.

Structures of various ABC transporter genes in bacteria such as *Escherichia coli* have been analyzed, and it is known that each gene coding for constituent of an ABC transporter forms an operon. In coryneform bacteria, however, most of genes coding for ABC transporters or constituents thereof involved in transport of amino acids across membranes remain unknown.

Disclosure of the Invention

The inventors of the present invention cloned a gene coding for an enzyme involved in one of L-glutamic acid biosynthetic pathways, glutamine-oxoglutarate aminotransferase (also referred to as glutamate synthase,

abbreviated as "GOGAT" hereinafter) for the purpose of breeding of coryneform bacteria producing L-glutamic acid. In this process, the inventors accidentally found that a DNA fragment containing a gene coding for GOGAT (*gltBD*) contained a gene coding for an ABC transporter considered to be involved in transport of amino acids, and thus accomplished the present invention.

That is, the present invention provides a protein, which is a constituent of ABC transporter, and a DNA coding for it.

A first constituent of ABC transporter according to the present invention is a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 8 shown in Sequence Listing;

(B) a protein which has the amino acid sequence of SEQ ID NO: 8 shown in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and constitutes an ABC transporter.

A second constituent of ABC transporter according to the present invention is a protein defined in the following (C) or (D):

(C) a protein which has the amino acid sequence of SEQ ID NO: 9 shown in Sequence Listing;

(D) a protein which has the amino acid sequence of SEQ ID NO: 9 shown in Sequence Listing including

substitution, deletion, insertion, addition or inversion of one or several amino acids, and has ATPase activity of ABC transporter.

A third constituent of ABC transporter according to the present invention is a protein defined in the following (E) or (F):

(E) a protein which has the amino acid sequence of SEQ ID NO: 10 shown in Sequence Listing;

(F) a protein which has the amino acid sequence of SEQ ID NO: 10 shown in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and constitutes an ABC transporter.

The present invention also provides DNAs coding for the aforementioned proteins that are constituents of ABC transporter.

The present invention further provides an operon coding for an ABC transporter.

Hereafter, the present invention will be explained in detail.

The DNA of the present invention was found from *Brevibacterium lactofermentum* as an ORF present in the neighborhood of the *gltBD* gene and can be obtained as follows.

PCR (polymerase chain reaction) is performed by using chromosomal DNA of *Brevibacterium lactofermentum* such as *Brevibacterium lactofermentum* ATCC13869 as a

template and primers having nucleotide sequences of regions in the *gltBD* genes of *Escherichia Coli* K-12 (*Gene, vol. 60,* pp.1-11 (1987) and yeast (*Saccharomyces cerevisiae*, GenBank Accession No. X89221) exhibiting high homology, for example, those having nucleotide sequences of SEQ ID NOS: 1 and 2 shown in Sequence Listing, to obtain a DNA fragment of about 1.4 kb. *Brevibacterium lactofermentum* ATCC13869 can be obtained from ATCC (the American Type Culture Collection: 12301 Parklawn Drive, Rockville, Maryland 20852, United States of America).

Subsequently, colony hybridization of a chromosomal DNA library of *Brevibacterium lactofermentum* ATCC13869 is performed by using the PCR-amplified fragment obtained as described above as a probe to obtain a DNA fragment hybridizable with the probe. Thus, the DNA of the present invention can be obtained together with the *gltBD* gene. If chromosomal DNA digested with *Hin*dIII is used in the preparation of the chromosomal DNA library, the DNA fragment can be obtained as a fragment of about 14 kb in length.

The above DNA fragment contains the *gltBD* gene and two open reading frames (ORFs) downstream the *gltBD* gene in the inverted direction with respect to the *gltBD* gene from the end. These ORFs correspond to the second ORF and third ORF, respectively, among the ORFs included in the nucleotide sequence of SEQ ID NO: 7.

As shown in examples described later, it is possible that the aforementioned two ORFs form an operon together with another ORF that exists upstream from them. This ORF corresponds to the first ORF among the ORFs included in the nucleotide sequence of SEQ ID NO: 7. This first ORF can be obtained as a DNA fragment of about 1.8 kb by PCR using chromosomal DNA of *Brevibacterium lactofermentum*, for example, the *Brevibacterium lactofermentum* ATCC13869, as a template and nucleotide sequences of SEQ ID NOS: 5 and 6 shown in Sequence Listing as primers. In this DNA fragment, a region estimated to be a promoter region exists in the upstream of the target ORF.

The nucleotide sequence shown in SEQ ID NO: 7 is obtained by ligating a nucleotide sequence (1.3 kb) in the aforementioned DNA fragment of about 14 kb with a nucleotide sequence (1.1 kb) in the aforementioned DNA fragment of about 1.8 kb.

Since the nucleotide sequences of the above ORFs and nucleotide sequences of flanking regions have been revealed, the above ORFs can also be obtained by PCR using oligonucleotides prepared based on such nucleotide sequences as primers.

Usual methods well known to those skilled in the art can be employed for preparation of chromosomal DNA, construction of chromosomal DNA library, hybridization, PCR, preparation of plasmid DNA, digestion and ligation

of DNA, transformation, design of oligonucleotides to be used as primers and so forth. These methods are described in Sambrook, J., Fritsch, E.F., Maniatis, T., Molecular Cloning, Cold Spring Harbor Laboratory Press, 1.21 (1989) and so forth.

The aforementioned second ORF and amino acid sequence encoded thereby were compared with known sequences for homology. The used databases were EMBL and SWISS-PROT. As a result, these sequences exhibited homology to already reported ATPase proteins constituting ABC transporters responsible for transport of the amino acids listed in Table 1 and genes coding for them. It is possible that the three ORFs containing these sequences form an operon.

Table 1

Gene	Substance to be tranported	Origin	Reference	Homology
artP	Arginine	*E. coli*	J.Bacteriol.175: 3687-3688 (1993)	31.0%
artP	Arginine	*Haemophilus Influenzae*	Science 269: 496-512 (1995)	31.8%
glnQ	Glutamine	*Bacillus Stearothermophilus*	J.Bacteriol.173: 4877-4888 (1991)	35.4%
glnQ	Glutamine	*E. coli*	Mol.Gen.Genet.205: 260-269 (1986)	33.5%
GltL	Glutamic acid/ Aspartic acid	*E. coli*	GeneBank Accession No.U10981	33.5%
gltL	Glutamic acid/ Aspartic acid	*Haemophilus influenzae*	Science 269: 496-512 (1995)	31.2%
gluA	Glutamic acid	*Corynebacterium glutamicum*	J.Bacteriol.177: 1152-1158	34.4%
hisP	Histidine	*E. coli*	Nature 298: 723-727 (1982)	33.0%
hisP	Histidine	*Salmonella typhimurium*	Nucleic acids Res.15: 8568-8568	34.4%

The gene coding for a constituent of ABC transporter according to the present invention may be one coding for an ATP-binding protein including substitution, deletion, insertion, addition or inversion of one or several amino acids at one or a plurality of positions so long as characteristics of the encoded protein are not deteriorated. The number meant by the term "several" used herein may vary depending on locations of amino acid residues in the three-dimensional structure of proteins and kinds of amino acid residues. This is due to the fact that there are highly analogous amino acids among amino acids such as isoleucine and valine, and difference among such amino

acids does not substantially affect the three-dimensional structure of proteins.

Such a DNA encoding a protein substantially the same as a constituent of ABC transporter as mentioned above can be obtained by modifying a nucleotide sequence by, for example, site-directed mutagenesis so that the amino acid residues of a specific site should include substitution, deletion, insertion, addition or inversion. Such a modified DNA as mentioned above can also be obtained by an already known mutagenesis treatment. Examples of the mutagenesis treatment include *in vitro* treatment of DNA coding for each protein with hydroxylamine etc., treatment of a microorganism having DNA coding for each protein, for example, genus *Escherichia*, by ultraviolet irradiation or with a mutagenizing agent used for usual mutagenesis treatment such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or nitrous acid.

The substitution, deletion, insertion, addition or inversion of nucleotides described above also includes mutations (mutant or variant) that naturally occurring due to individual difference, difference in species or genera of a microorganism having each constituent.

A DNA coding for substantially the same protein as a constituent of ABC transporter can be obtained by expressing DNA having such a mutation as described above in an appropriate cell, and examining characteristics of

an expressed product. A DNA coding for substantially the same protein as a constituent of ABC transporter can also be obtained by isolating a DNA hybridizable with a nucleotide sequence coding for each constituent or a probe prepared from such a nucleotide sequence, for example, the nucleotide sequence of nucleotide numbers 1117 to 1725 in SEQ ID NO: 7 or a probe prepared from this nucleotide sequence, for ATPase under a stringent condition, and coding for a protein having the characteristics of the constituent from a DNA coding for each protein having mutation or from a cell harboring it. The "stringent condition" referred to herein is a condition under which a so-called specific hybrid is formed, but a non-specific hybrid is not formed. It is difficult to clearly define this condition by using numerical values. However, for example, the stringent condition includes a condition under which two of DNAs having high homology, for example, two of DNAs having homology of not less than 40% are hybridized with each other, but two of DNAs having homology lower than the above level are not hybridized with each other. Alternatively, the stringent condition is exemplified by a hybridization condition represented by salt concentrations of 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C, which is an ordinary condition of washing in Southern hybridization.

Those genes hybridizable under the condition as

described above include those having a stop codon generated in the genes, and those having no activity due to mutation of the active center. However, such mutant genes can be easily removed by using a commercially available activity expression vector to examine the characteristics of the expressed product.

The DNA coding for a constituent of ABC transporter according to the present invention and an operon of ABC transporter (hereafter, these may be referred to simply as "gene of the present invention") can be utilized in breeding of coryneform bacteria. That is, since the ABC transporter of the present invention or a constituent thereof is considered to be involved in transport of amino acids, characteristics of a cell concerning transport of amino acids can be modified by modifying expression of these genes.

Coryneform bacteria to which the present invention is applicable include those bacteria having been hitherto classified into the genus *Brevibacterium* but united into the genus *Corynebacterium* at present (*Int. J. Syst. Bacteriol.*, *41*, 255 (1981)), and include bacteria belonging to the genus *Brevibacterium* closely relative to the genus *Corynebacterium*. Examples of such coryneform bacteria are mentioned below.

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium alkanolyticum

Corynebacterium callunae

Corynebacterium glutamicum

Corynebacterium lilium (*Corynebacterium glutamicum*)

Corynebacterium melassecola

Corynebacterium thermoaminogenes

Corynebacterium herculis

Brevibacterium divaricatum (*Corynebacterium glutamicum*)

Brevibacterium flavum (*Corynebacterium glutamicum*)

Brevibacterium immariophilum

Brevibacterium lactofermentum (*Corynebacterium glutamicum*)

Brevibacterium roseum

Brevibacterium saccharolyticum

Brevibacterium thiogenitalis

Brevibacterium album

Brevibacterium cerium

Microbacterium ammoniaphilum

Specifically, the following strains can be exemplified.

Corynebacterium acetoacidophilum ATCC 13870

Corynebacterium acetoglutamicum ATCC 15806

Corynebacterium alkanolyticum ATCC21511

Corynebacterium callunae ATCC 15991

Corynebacterium glutamicum ATCC 13020, 13032, 13060

Corynebacterium lilium (*Corynebacterium glutamicum*) ATCC 15990

Corynebacterium melassecola ATCC 17965

Corynebacterium thermoaminogenes AJ12340 (FERM BP-1539)

Corynebacterium herculis ATCC13868

Brevibacterium divaricatum (*Corynebacterium glutamicum*) ATCC 14020

Brevibacterium flavum (*Corynebacterium glutamicum*) ATCC 13826, ATCC 14067

Brevibacterium immariophilum ATCC 14068

Brevibacterium lactofermentum (*Corynebacterium glutamicum*) ATCC 13665, ATCC 13869

Brevibacterium roseum ATCC 13825

Brevibacterium saccharolyticum ATCC 14066

Brevibacterium thiogenitalis ATCC 19240

Brevibacterium album ATCC15111

Brevibacterium cerium ATCC15112

Microbacterium ammoniaphilum ATCC15354

Methods of modifying a gene coding for an ABC transporter or a constituent thereof include amplification or disruption of the gene. The gene or the like can be amplified by transforming a coryneform bacterium with a recombinant vector obtained by ligating the gene to a vector such as a plasmid. At this time, amplification efficiency can be improved by using a multiple copy type vector. Examples of such a vector

include plasmids autonomously replicable in coryneform bacterium including those mentioned below.

pAM330 (refer to Japanese Patent Laid-Open (Kokai) No. 58-67699)

pHM1519 (refer to Japanese Patent Laid-Open No. 58-77895)

pAJ655 (refer to Japanese Patent Laid-Open No. 58-192900)

pAJ611 (refer to Japanese Patent Laid-Open No. 58-192900)

pAJ1844 (refer to Japanese Patent Laid-Open No. 58-192900)

pCG1 (refer to Japanese Patent Laid-Open No. 57-134500)

pCG2 (refer to Japanese Patent Laid-Open No. 58-35197)

pCG4 (refer to Japanese Patent Laid-Open No. 57-183799)

pCG11 (refer to Japanese Patent Laid-Open No. 57-183799)

Coryneform bacteria can be transformed by the electric pulse method (refer to Japanese Patent Laid-Open No. 2-207791).

The gene can also be amplified by allowing multiple copies of the gene of the present invention to exist on chromosomal DNA of a host such as those mentioned above. Multiple copies of a target gene can

be introduced into chromosomal DNA of coryneform bacterium by homologous recombination utilizing multiple copies of sequences existing on chromosomal DNA as targets (Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press (1972); Matsuyama, S. and Mizushima, S., *J. Bacteriol.*, *162*, 1196 (1985)). As sequences of which multiple copies exist on the chromosomal DNA, repetitive DNA and inverted repeats that exist at an end of transposable element can be used. As disclosed in Japanese Patent Laid-open No. 2-109985, it is also possible to insert the target gene into transposon, and allow it to transfer to introduce multiple copies thereof into the chromosomal DNA.

Further, expression of the gene can be modified by replacing an expression regulatory sequence of the gene originally present on a chromosome, such as a promoter, with a stronger one or one having weak functions.

Moreover, gene disruption methods by homologous recombination have already been established, and the gene can be disrupted by a method using linear DNA or a temperature sensitive plasmid.

Best Mode for Carrying out the Invention

Hereafter, the present invention will be explained in more detail with reference to the following examples.

(1) Cloning of *gltBD* gene of *Brevibacterium lactofermentum* ATCC13869

A region of *gltB* gene products of *Escherichia coli* and yeast showing high homology for amino acid sequence was selected, and a nucleotide sequence was deduced from the sequence, oligonucleotides shown as SEQ ID NOS: 1 and 2 were synthesized. Separately, chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 was prepared by using a Bacterial Genomic DNA Purification Kit (produced by Advanced Genetic Technologies Corp.). PCR was performed by using this chromosomal DNA as a template and the oligonucleotides as primers under the standard reaction conditions described in "PCR Technology", p. 8, Ed. by Henry Ehrlich, Stockton Press, 1989. The PCR product was subjected to agarose gel electrophoresis, and it was found that a DNA fragment of about 1.4 kb was amplified.

The obtained DNA was sequenced for the nucleotide sequences of the both ends by using the oligonucleotides of SEQ ID NOS: 1 and 2. The nucleotide sequencing was performed according to the method of Sanger (*J. Mol. Biol.*, *143*, 161 (1980)) by using a DNA Sequencing Kit (produced by Applied Biosystems Co.). The determined nucleotide sequence was translated into an amino acid sequence, and compared with an amino acid sequence deduced from the *gltB* gene of *Escherichia coli* and yeast. As a result, high homology was observed. Therefore, it

was determined that the DNA fragment amplified by the PCR should be a part of the gltB gene of *Brevibacterium lactofermentum* ATCC13869. By using this PCR-amplified DNA fragment as a probe and a DIG DNA Labeling and Detection Kit (produced by Boehringer Manheim), fragments obtained by digesting chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 prepared by the above method with *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I or *Sal*I (produced by Takara Shuzo Co., Ltd.) were subjected to Southern hybridization in a conventional manner. AS a result, it was found that a fragment of 14 kb digested with *Hin*dIII was hybridized with the probe DNA.

Then, the *Hin*dIII fragment of chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 prepared in a conventional manner was subjected to agarose electrophoresis and a DNA fragment of about 10 kb or longer was recovered by using glass powder. The recovered DNA fragments and vector pMW219 (produced by Nippon Gene) digested with a restriction enzyme, *Hin*dIII (produced by Takara Shuzo Co., Ltd.), were ligated by using a ligation kit (produced by Takara Shuzo Co., Ltd.), and used for transformation of competent cells of *Escherichia coli* JM109 (produced by Takara Shuzo Co., Ltd.). The transformant strains were plated on L medium (10 g/L of Bacto trypton, 5 g/L of Bacto yeast extract, 5 g/L of NaCl, and 15 g/L of agar, pH 7.2) containing 10 μg/ml of IPTG (isopropyl-â-D-thiogalactopyranoside), 40

μg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-â-D-galactoside) and 25 μg/ml of kanamycin, and cultured overnight. Then, the appeared white colonies were picked up and separated into single colonies to obtain about 1,000 transformants.

Plasmids were prepared from the obtained transformant strains by using the alkaline method (Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, p.105, Baifukan, 1992). PCR was performed under the above conditions by using as primers synthetic oligonucleotides of nucleotide sequences shown as SEQ ID NOS: 3 and 4, which were prepared based on the sequenced portion in the DNA used as a probe, and the plasmids as a template. Then, there was selected a transformant harboring a plasmid with which an amplified fragment having the same length as the DNA fragment amplified by PCR using these primers and chromosome of *Brevibacterium lactofermentum* ATCC13869 as a template, that is, about 1.3 kb, could be obtained.

(2) Sequencing of DNA fragment containing *Brevibacterium lactofermentum* ATCC13869 *gltBD* gene for total nucleotide sequence and isolation of ABC transporter gene

The plasmid DNA prepared by the alkaline method from the transformant obtained in the above (1) contained a DNA fragment of about 14 kb derived from a

Brevibacterium lactofermentum ATCC13869 chromosome. The DNA fragment of about 14 kb derived from the *Brevibacterium lactofermentum* ATCC13869 chromosome in the obtained plasmid was sequenced for the total nucleotide sequence in the same manner as the method described above. As a result, it was found that, while the obtained DNA fragment contained the *gltBD* gene in the full length, it also contained two open reading frames of 500 bps or longer downstream from the *gltBD* gene in an inverted direction from the end and a sequence estimated to be a terminater downstream from these open reading frames. However, since these open reading frames lacked a promoter region, a region upstream from them was cloned as described below.

The region was cloned from a DNA fragment obtained through digestion of chromosome of *Brevibacterium lactofermentum* ATCC13869 with a restriction enzyme *Bam*HI by using primers of SEQ ID NOS: 5 and 6 shown in Sequence Listing and an LA PCR in vitro cloning Kit (produced by Takara Shuzo Co., Ltd.). As a result of PCR performed by using the aforementioned primers, a DNA fragment of about 1.8 kb was amplified, and hence this DNA fragment was sequenced for the nucleotide sequence in the same manner as described above. As a result, it was found that the amplified DNA fragment contained an open reading frame for about 350 amino acids located upstream from the aforementioned two open reading frames

and a region estimated to be a promoter region further upstream from it. Therefore, it is possible that these three open reading frames consititute an operon.

Nucleotide sequences of these open reading frames are shown in SEQ ID NO: 7 in Sequence Listing. Amino acid sequences of products deduced from the nucleotide sequences were also shown in SEQ ID NO: 7 in Sequence Listing. Among these, the nucleotide numbers 1 to 1101 represent the first open reading frame, the nucleotide numbers 1117 to 1725 represent the second open reading frame and the nucleotide numbers 1759 to 2367 represent the third open reading frame. A methionine residue present at the N-terminus of the protein encoded by each open reading frame was derived from the initiation codon. It is well known that such a methionine residue may be usually irrelevant to function of the protein and eliminated by the action of peptidase after the translation. In the case of the aforementioned proteins, the methionine residue at the N-terminus may also be eliminated. Further, since the promoter region and terminater sequence estimated above were obtained just as a result of computarized analyses, it is possible that open reading frames may be present upstream or downstream from them and expressed together with them in fact.

The nucleotide sequences and amino acid sequences were compared with known sequences for homology. The

used database were EMBL and SWISS-PROT. As a result, it was found that DNA shown as SEQ ID NO: 7 in Sequence Listing and proteins encoded by it were novel genes and proteins for bacteria belonging to the genus *Corynebacterium*. It was found that, among these, the second open reading frame and the protein encoded by it showed high homology to the already reported ATP-binding proteins of ABC transporters and the genes coding for them, and it was a gene coding for an ATP-binding protein that was novel for bacteria belonging to the genus *Corynebacterium*.

Industrial Applicability

According to the present invention, constituents of ABC transporters of *Brevibacterium lactofermentum* and DNA coding for them are provided. The genes of the present invention can be utilized for breeding of coryneform bacteria.

What is claimed is:

1. A protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 8 shown in Sequence Listing;

(B) a protein which has the amino acid sequence of SEQ ID NO: 8 shown in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and constitutes an ABC transporter.

2. A DNA which codes for a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 8 shown in Sequence Listing;

(B) a protein which has the amino acid sequence of SEQ ID NO: 8 shown in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and constitutes an ABC transporter.

3. The DNA according to Claim 2, which is a DNA defined in the following (a) or (b):

(a) a DNA which comprises the nucleotide sequence of nucleotide numbers 1 to 1101 of SEQ ID NO: 7 shown in Sequence Listing;

(b) a DNA which is hybridizable with the nucleotide sequence of nucleotide numbers 1 to 1101 of SEQ ID NO: 7 or a probe prepared from the nucleotide sequence under a stringent condition, and codes for a

protein constituting an ABC transporter.

4. The DNA according to Claim 3, wherein the stringent condition is a condition in which washing is performed at 60°C and a salt concentration corresponding to 1 x SSC and 0.1 % SDS.

5. A protein defined in the following (C) or (D):

(C) a protein which has the amino acid sequence of SEQ ID NO: 9 shown in Sequence Listing;

(D) a protein which has the amino acid sequence of SEQ ID NO: 9 shown in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has ATPase activity of ABC transporter.

6. A DNA coding for a protein defined in the following (C) or (D):

(C) a protein which has the amino acid sequence of SEQ ID NO: 9 shown in Sequence Listing;

(D) a protein which has the amino acid sequence of SEQ ID NO: 9 shown in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has ATPase activity of ABC transporter.

7. The DNA according to Claim 6, which is a DNA defined in the following (c) or (d):

(c) a DNA which comprises the nucleotide sequence of nucleotide numbers 1117 to 1725 of SEQ ID NO: 7 shown in Sequence Listing;

(d) a DNA which is hybridizable with the nucleotide sequence of nucleotide numbers 1117 to 1725 of SEQ ID NO: 7 or a probe prepared from the nucleotide sequence under a stringent condition, and codes for a protein having ATPase activity of ABC transporter.

8. The DNA according to Claim 7, wherein the stringent condition is a condition in which washing is performed at 60°C and a salt concentration corresponding to 1 x SSC and 0.1% SDS.

9. A protein defined in the following (E) or (F):

(E) a protein which has the amino acid sequence of SEQ ID NO: 10 shown in Sequence Listing;

(F) a protein which has the amino acid sequence of SEQ ID NO: 10 shown in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and constitutes an ABC transporter.

10. A DNA coding for a protein defined in the following (E) or (F):

(E) a protein which has the amino acid sequence of SEQ ID NO: 10 shown in Sequence Listing;

(F) a protein which has the amino acid sequence of SEQ ID NO: 10 shown in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and constitutes an ABC transporter.

11. The DNA according to Claim 10, which is a DNA

defined in the following (e) or (f):

(e) a DNA which comprises the nucleotide sequence of nucleotide numbers 1759 to 2367 of SEQ ID NO: 7 shown in Sequence Listing;

(f) a DNA which is hybridizable with the nucleotide sequence of nucleotide numbers 1759 to 2367 of SEQ ID NO: 7 or a probe prepared from the nucleotide sequence under a stringent condition, and codes for a protein constituting an ABC transporter.

12. The DNA according to Claim 11, wherein the stringent condition is a condition in which washing is performed at 60°C and at a salt concentration corresponding to 1 x SSC and 0.1% SDS.

13. A DNA which comprises a nucleotide sequence coding for a protein having the amino acid sequence of SEQ ID NO: 8, a nucleotide sequence coding for a protein having the amino acid sequence of SEQ ID NO: 9 and a nucleotide sequence coding for a protein having the amino acid sequence of SEQ ID NO: 10.

14. The DNA according to Claim 13, which has the nucleotide sequence shown as SEQ ID NO: 7.

Abstract of the Disclosure

The present invention provides constituents of ABC transporter of *Brevibacterium lactofermentum* having an amino acid sequence of SEQ ID NO: 8, 9 or 10 shown in Sequence Listing and DNAs coding for them. The DNA of the present invention can be utilized for breeding of coryneform bacteria.

Sequence Listing

<110> KANNO, Sohei
KIMURA, Eiichiro
MATSUI, Kazuhiko
NAKAMATSU, Tsuyoshi

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<141> 1999-12-16

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amplifying Brevibacterium lactofermentum gltBD gene

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Ser Phe Lys Lys Ser Lys Lys Thr Met Gly Ser Ala Ser Thr Arg Lys
275 280 285
Ser Ser Ile Lys Ser Ser Ile Thr Ala Ala Asn Cys Arg Ser Asn Phe
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Arg Phe Asn Ser Val Arg Leu Ala Arg Glu Pro Leu Phe Arg Ala Ile
305 310 315 320
Cys Ser Asn Ser Phe Arg Cys Ser Leu Ser Gln Ile Leu Arg Thr Ser
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        35                  40                  45
Thr Leu Asp Lys Pro Ser Ser Gly Gln Ile Leu Val Glu Asp Val Asp
    50                  55                  60
Leu Leu Lys Leu Ser Thr Arg Lys Gln Arg Leu Tyr Arg Lys Asn Thr
65                  70                  75                  80
Val Gly Tyr Leu Phe Gln Asp Tyr Ala Leu Ile Pro Asp Arg Thr Val
                85                  90                  95
Lys Phe Asn Leu Gln Leu Ala Val Glu Lys His Lys Trp Pro Glu Ile
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Pro Gln Val Leu His Ala Val Gly Leu Glu Ser Phe Glu Glu Lys Pro
        115                 120                 125
Val Phe Glu Leu Ser Gly Gly Glu Gln Gln Arg Thr Ala Leu Ala Arg
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Val Leu Leu Lys Asn Pro Arg Ile Ile Leu Ala Asp Glu Pro Thr Gly
145                 150                 155                 160
Ala Leu Asp Leu Thr Asn Ser Glu Leu Val Ile Glu Ala Leu Arg Ala
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Val Leu Cys Phe Ile Pro Lys Leu Ser Val Pro Phe Val Val Leu Leu
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Ser Ile Ala Gln Leu Ala Leu Ala Tyr Leu Cys Ile His Gly Glu Pro
65                  70                  75                  80
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Gly Leu Leu Met Phe Arg Gly Gln Arg Val Leu Ala Phe Ile Ser Ala
            100                 105                 110
Gly Gly Leu Ile Trp Ile Gly Thr Ile Asp Pro Thr Asn Gly Ala Trp
        115                 120                 125
Ser Pro His Val Met Ser Ala Leu Ala Leu Ala Val Phe Phe Ala Leu
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Asn Leu Glu Glu Gln Ala Lys Ile Gln Thr Glu Leu Arg Arg Lys Glu
                165                 170                 175
Leu Ser Thr Pro Ser Ala Ser Val Gly Cys Gln Arg Thr Tyr Val Cys
            180                 185                 190
Ser Asp Glu Ile Ala Gly Ala Gln Trp Ser Arg
        195                 200
```

Declaration, Power Of Attorney and Petition

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ABC TRANSPORTER AND GENE CODING FOR THE SAME

the specification of which

☒ is attached hereto.

☐ was filed on _____ as

Application Serial No. _____

and amended on _____.

☒ was filed as PCT international application

Number PCT/JP 99/07079

on December 16, 1999,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
10-360621	Japan	18/12/1998	☒ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)

(25)

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; Timothy R. Schwartz, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Goolkasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; and Richard L. Chinn, Registration Number 34,305; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1-00

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Date

2-00

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Date

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June 5, 2001

Date

NAME OF FIFTH JOINT INVENTOR

Signature of Inventor

Date

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MATSUI, KAZUHIKO
NAKAMATSU, TSUYOSHI
KIMURA, EIICHIRO

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1               5                  10                  15

gga tcg cta acc ggt aac ctc agt gaa cta cgt gca caa act act ttt       96
Gly Ser Leu Thr Gly Asn Leu Ser Glu Leu Arg Ala Gln Thr Thr Phe
            20                  25                  30

agt aca tta tgg gat acc cat aaa gaa acc tat aga gtc tcc ata gct      144
Ser Thr Leu Trp Asp Thr His Lys Glu Thr Tyr Arg Val Ser Ile Ala
        35                  40                  45

tcc gca gca gga caa gac ttc tac ggg ctt gct gag act cta cgc act      192
Ser Ala Ala Gly Gln Asp Phe Tyr Gly Leu Ala Glu Thr Leu Arg Thr
    50                  55                  60

atg gat agg cat ggg gaa att att ttg gca gat cgt caa tgg tta aca      240
Met Asp Arg His Gly Glu Ile Ile Leu Ala Asp Arg Gln Trp Leu Thr
65                  70                  75                  80

gct ccc ctt gat atc ggt gca cca gtc gta tta tca aac aca act ttt      288
Ala Pro Leu Asp Ile Gly Ala Pro Val Val Leu Ser Asn Thr Thr Phe
                85                  90                  95

gcc gtt gat gaa gga cta ctt gcg cca aaa gat cta ccg caa agt gac      336
Ala Val Asp Glu Gly Leu Leu Ala Pro Lys Asp Leu Pro Gln Ser Asp
            100                 105                 110
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Glu Ile Thr Ile Leu His Pro Gln Phe Leu Asp Ser Ala Lys Glu Pro
        115                 120                 125

gaa tta ctt ggt ttg ctg gag ttc gaa gca tcc aac tca caa gtg cca      432
Glu Leu Leu Gly Leu Leu Glu Phe Glu Ala Ser Asn Ser Gln Val Pro
    130                 135                 140

atg cca aag atc caa agc att cca tat gat agc gaa gac tca acc aac      480
Met Pro Lys Ile Gln Ser Ile Pro Tyr Asp Ser Glu Asp Ser Thr Asn
145                 150                 155                 160

ccc atg tct gaa gtt ttt acc tac aac att aac ctg gat agt gca gta      528
Pro Met Ser Glu Val Phe Thr Tyr Asn Ile Asn Leu Asp Ser Ala Val
                165                 170                 175

aga aac cca atc gta gtt atc ctt ccc gca ggc tta gag ctt tta agt      576
Arg Asn Pro Ile Val Val Ile Leu Pro Ala Gly Leu Glu Leu Leu Ser
            180                 185                 190

gat caa aat ttg tcg gct cga ctc aca cag aat agt ctg ctg ata aaa      624
Asp Gln Asn Leu Ser Ala Arg Leu Thr Gln Asn Ser Leu Leu Ile Lys
        195                 200                 205

gac cag act ggt gtg aac gct ctt cta tcc tca gag gat tca cgc aat      672
Asp Gln Thr Gly Val Asn Ala Leu Leu Ser Ser Glu Asp Ser Arg Asn
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tat gtg gga gct gca tcc ccg atg att gac acg tgg gaa gaa agc gtt      720
Tyr Val Gly Ala Ala Ser Pro Met Ile Asp Thr Trp Glu Glu Ser Val
225                 230                 235                 240

gtt cgg ttg aag gaa gcg aac caa ata atc gcc ttc aac gct ttc att      768
Val Arg Leu Lys Glu Ala Asn Gln Ile Ile Ala Phe Asn Ala Phe Ile
                245                 250                 255

gca ttg ttc ctc acg acg act ctt gtt cta gca tac tgc act ggt att      816
Ala Leu Phe Leu Thr Thr Thr Leu Val Leu Ala Tyr Cys Thr Gly Ile
            260                 265                 270

tca ttt aag aaa tca aag aag act atg ggt agc gca tct act agg aaa      864
Ser Phe Lys Lys Ser Lys Lys Thr Met Gly Ser Ala Ser Thr Arg Lys
        275                 280                 285

tca tcc att aag agc tcg att aca gct gct aat tgt aga agt aat ttt      912
Ser Ser Ile Lys Ser Ser Ile Thr Ala Ala Asn Cys Arg Ser Asn Phe
    290                 295                 300

cgc ttc aat tcc gtg cgt ctg gct cgc gaa ccg cta ttt cga gcg atc      960
Arg Phe Asn Ser Val Arg Leu Ala Arg Glu Pro Leu Phe Arg Ala Ile
305                 310                 315                 320

tgc agc aat agc ttc aga tgc tcc ctc agc cag ata ctt aga aca tct     1008
Cys Ser Asn Ser Phe Arg Cys Ser Leu Ser Gln Ile Leu Arg Thr Ser
                325                 330                 335
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Gln Phe Tyr Thr Ser Ile Thr Ala Val Gly Phe Arg Asn Leu Asn Asn
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cgg ttg gac ttc act ttc att ttt cag ttc gat gaa gct tcc ttt           1101
Arg Leu Asp Phe Thr Phe Ile Phe Gln Phe Asp Glu Ala Ser Phe
        355                 360                 365

tgaaaagagc acaca atg ata gaa atc aat gac ctc aag aaa tct ttt ggc     1152
                 Met Ile Glu Ile Asn Asp Leu Lys Lys Ser Phe Gly
                         370                 375

gtt cgg atc tta tgg caa ggt ctc agt cat aag ttt tta cca gga aca       1200
Val Arg Ile Leu Trp Gln Gly Leu Ser His Lys Phe Leu Pro Gly Thr
380                 385                 390                 395

atg aca gca ctg act gga gcg tcc ggt tca gga aaa tcg act ttg ctc       1248
Met Thr Ala Leu Thr Gly Ala Ser Gly Ser Gly Lys Ser Thr Leu Leu
                400                 405                 410

aac tgt ctt ggc aca ctt gac aaa cca agt tcc gga cag atc ctt gtc       1296
Asn Cys Leu Gly Thr Leu Asp Lys Pro Ser Ser Gly Gln Ile Leu Val
            415                 420                 425

gag gat gta gac ctt ctg aaa ctc tct acg cgt aag caa cgg tta tac       1344
Glu Asp Val Asp Leu Leu Lys Leu Ser Thr Arg Lys Gln Arg Leu Tyr
        430                 435                 440

agg aaa aat acg gtg ggc tat tta ttt caa gat tat gcc ttg att ccc       1392
Arg Lys Asn Thr Val Gly Tyr Leu Phe Gln Asp Tyr Ala Leu Ile Pro
    445                 450                 455

gac agg aca gtt aaa ttc aac ctt cag ctt gcg gtg gaa aaa cac aaa       1440
Asp Arg Thr Val Lys Phe Asn Leu Gln Leu Ala Val Glu Lys His Lys
460                 465                 470                 475

tgg cct gaa att cct caa gta ctt cat gct gtt ggt ctt gag tcg ttc       1488
Trp Pro Glu Ile Pro Gln Val Leu His Ala Val Gly Leu Glu Ser Phe
                480                 485                 490

gag gaa aag cca gtt ttt gaa ctc tct ggt ggc gaa caa caa cga act       1536
Glu Glu Lys Pro Val Phe Glu Leu Ser Gly Gly Glu Gln Gln Arg Thr
            495                 500                 505

gcg ttg gcc cgg gta ctg ctc aaa aat ccc cga ata att ctg gct gat       1584
Ala Leu Ala Arg Val Leu Leu Lys Asn Pro Arg Ile Ile Leu Ala Asp
        510                 515                 520

gaa cca acc gga gct cta gat tta aca aac agt gag cta gtc ata gaa       1632
Glu Pro Thr Gly Ala Leu Asp Leu Thr Asn Ser Glu Leu Val Ile Glu
    525                 530                 535

gca ttg aga gca ctc gcc gac aaa ggc gcc acc gtt gtt gtt gct acg       1680
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cac tcg ccc ctc ttc cga gaa tca gcg gat acc att atc aaa cta           1725
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His Ser Pro Leu Phe Arg Glu Ser Ala Asp Thr Ile Ile Lys Leu
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                                    Met Met Glu Phe Leu Asn Thr
                                                        575

cac cgt ttg att gtt ctc ggg agt ttg tct ttt cta ggg cta ggt ttc    1827
His Arg Leu Ile Val Leu Gly Ser Leu Ser Phe Leu Gly Leu Gly Phe
        580                 585                 590

gcg gaa gtc ctg ctg cgt ggc cag tgg tca aca ccg cag ttt ttt gtt    1875
Ala Glu Val Leu Leu Arg Gly Gln Trp Ser Thr Pro Gln Phe Phe Val
    595                 600                 605

ttc act ttc ttg caa act ctg ctt ctc gta ttg tgt ttt att cct aaa    1923
Phe Thr Phe Leu Gln Thr Leu Leu Leu Val Leu Cys Phe Ile Pro Lys
610                 615                 620                 625

ctc tcg gtt cct ttt gtg gtg ctt cta agc att gcc caa ctc gcg ctt    1971
Leu Ser Val Pro Phe Val Val Leu Leu Ser Ile Ala Gln Leu Ala Leu
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gca tac ctg tgt att cat ggt gaa cct caa agc acc agc cct ttc act    2019
Ala Tyr Leu Cys Ile His Gly Glu Pro Gln Ser Thr Ser Pro Phe Thr
            645                 650                 655

tta att gtt gcc caa atg gcg ttt tcg gga ttg ctc atg ttc aga ggg    2067
Leu Ile Val Ala Gln Met Ala Phe Ser Gly Leu Leu Met Phe Arg Gly
        660                 665                 670

caa cgg gtg ctc gct ttt atc tct gca ggt ggg ctc att tgg att ggg    2115
Gln Arg Val Leu Ala Phe Ile Ser Ala Gly Gly Leu Ile Trp Ile Gly
    675                 680                 685

acc atc gat cca aca aac ggt gct tgg tct cct cat gtg atg tcc gcg    2163
Thr Ile Asp Pro Thr Asn Gly Ala Trp Ser Pro His Val Met Ser Ala
690                 695                 700                 705

cta gca ctt gcc gta ttc ttt gcg ctg tcg atg gca ctt gga cag gtt    2211
Leu Ala Leu Ala Val Phe Phe Ala Leu Ser Met Ala Leu Gly Gln Val
                710                 715                 720

ctt cga tca aaa gtt gaa caa aga gcc aac ctt gag gag cag gca aaa    2259
Leu Arg Ser Lys Val Glu Gln Arg Ala Asn Leu Glu Glu Gln Ala Lys
            725                 730                 735

att cag aca gaa ctg cgc aga aaa gaa cta agc act cca tct gca tcg    2307
Ile Gln Thr Glu Leu Arg Arg Lys Glu Leu Ser Thr Pro Ser Ala Ser
        740                 745                 750

gtc ggt tgc caa aga act tac gtt tgc agt gat gaa atc gca gga gct    2355
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Ser Thr Leu Trp Asp Thr His Lys Glu Thr Tyr Arg Val Ser Ile Ala
        35                  40                  45

Ser Ala Ala Gly Gln Asp Phe Tyr Gly Leu Ala Glu Thr Leu Arg Thr
    50                  55                  60

Met Asp Arg His Gly Glu Ile Ile Leu Ala Asp Arg Gln Trp Leu Thr
65                  70                  75                  80

Ala Pro Leu Asp Ile Gly Ala Pro Val Val Leu Ser Asn Thr Thr Phe
                85                  90                  95

Ala Val Asp Glu Gly Leu Leu Ala Pro Lys Asp Leu Pro Gln Ser Asp
            100                 105                 110

Glu Ile Thr Ile Leu His Pro Gln Phe Leu Asp Ser Ala Lys Glu Pro
        115                 120                 125

Glu Leu Leu Gly Leu Leu Glu Phe Glu Ala Ser Asn Ser Gln Val Pro
    130                 135                 140

Met Pro Lys Ile Gln Ser Ile Pro Tyr Asp Ser Glu Asp Ser Thr Asn
145                 150                 155                 160

Pro Met Ser Glu Val Phe Thr Tyr Asn Ile Asn Leu Asp Ser Ala Val
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Arg Asn Pro Ile Val Val Ile Leu Pro Ala Gly Leu Glu Leu Leu Ser
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Val Arg Leu Lys Glu Ala Asn Gln Ile Ile Ala Phe Asn Ala Phe Ile
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Ala Leu Phe Leu Thr Thr Thr Leu Val Leu Ala Tyr Cys Thr Gly Ile
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Ser Phe Lys Lys Ser Lys Lys Thr Met Gly Ser Ala Ser Thr Arg Lys
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Ser Ser Ile Lys Ser Ser Ile Thr Ala Ala Asn Cys Arg Ser Asn Phe
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Cys Ser Asn Ser Phe Arg Cys Ser Leu Ser Gln Ile Leu Arg Thr Ser
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Gln Phe Tyr Thr Ser Ile Thr Ala Val Gly Phe Arg Asn Leu Asn Asn
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Lys Phe Asn Leu Gln Leu Ala Val Glu Lys His Lys Trp Pro Glu Ile
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115 120 125

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145 150 155 160

Ala Leu Asp Leu Thr Asn Ser Glu Leu Val Ile Glu Ala Leu Arg Ala
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Phe Arg Glu Ser Ala Asp Thr Ile Ile Lys Leu
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Ser Thr Pro Gln Phe Phe Val Phe Thr Phe Leu Gln Thr Leu Leu Leu
        35                  40                  45

Val Leu Cys Phe Ile Pro Lys Leu Ser Val Pro Phe Val Val Leu Leu
    50                  55                  60

Ser Ile Ala Gln Leu Ala Leu Ala Tyr Leu Cys Ile His Gly Glu Pro
65                  70                  75                  80

Gln Ser Thr Ser Pro Phe Thr Leu Ile Val Ala Gln Met Ala Phe Ser
                85                  90                  95

Gly Leu Leu Met Phe Arg Gly Gln Arg Val Leu Ala Phe Ile Ser Ala
            100                 105                 110

Gly Gly Leu Ile Trp Ile Gly Thr Ile Asp Pro Thr Asn Gly Ala Trp
        115                 120                 125

Ser Pro His Val Met Ser Ala Leu Ala Leu Ala Val Phe Phe Ala Leu
    130                 135                 140

Ser Met Ala Leu Gly Gln Val Leu Arg Ser Lys Val Glu Gln Arg Ala
145                 150                 155                 160

Asn Leu Glu Glu Gln Ala Lys Ile Gln Thr Glu Leu Arg Arg Lys Glu
                165                 170                 175

Leu Ser Thr Pro Ser Ala Ser Val Gly Cys Gln Arg Thr Tyr Val Cys
            180                 185                 190

Ser Asp Glu Ile Ala Gly Ala Gln Trp Ser Arg
        195                 200
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